Student Research

Association of Urinary Levels of Estrogens and Estrogen Metabolites with Mammographic Density in Postmenopausal Women

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INTRODUCTION

Breast cancer is the most common and second deadliest cancer of women living in the United States. It is estimated that 232,340 new breast cancer cases in women will be diagnosed, and 39,620 women will lose their lives as a result of breast cancer in 2013.[1]

Currently, there is convincing evidence from epidemiological, animal, and in vitro studies that endogenous sex hormones, particularly estrogens, play a critical role in the etiology of breast cancer.[2] Sex steroids, including estrogens, promote cellular growth and proliferation,[3] and their circulating and urinary concentrations are elevated in hormone-dependent breast cancers.[4-6]

The metabolism of parent estrogens, estradiol and estrone, first starts with irreversible hydroxylation through the action of cytochrome P450 (CYP450) enzymes. This leads to the conversion of the parent estrogens to catechol estrogens including 2- and 4-hydroxyestradiols (2- and 4-OHE2) and 2- and 4-hydroxyestrones (2- and 4-OHE1), and 16-α-hydroxyestrone (16-α-OHE1). Catechol estrogens are further metabolized (methylated) to methoxyestrogens (e.g., 2- and 4- MeoE2, and 2- and 4- MeoE1) by the catechol-O-methyltransferase (COMT) enzyme (Figure 1). The COMT gene is polymorphic; a single G to A transition at codon 158 of COMT (SNP rs4680) results in a 3- to 4-fold decrease in enzymatic activity (GG vs. AA genotype). Also, individuals with heterozygous genotype (A/G) show intermediate levels of COMT activity.[7-8] Given the role of the COMT enzyme in the conversion of catechol estrogens to methoxyestrogens, any genetic variation in this enzyme might influence the risk of breast cancer as a result of significant changes in the estrogen metabolites levels.[9] Therefore, it is intriguing to speculate that individual genetic variability in the COMT enzyme may affect breast density and consequently influence breast cancer risk.

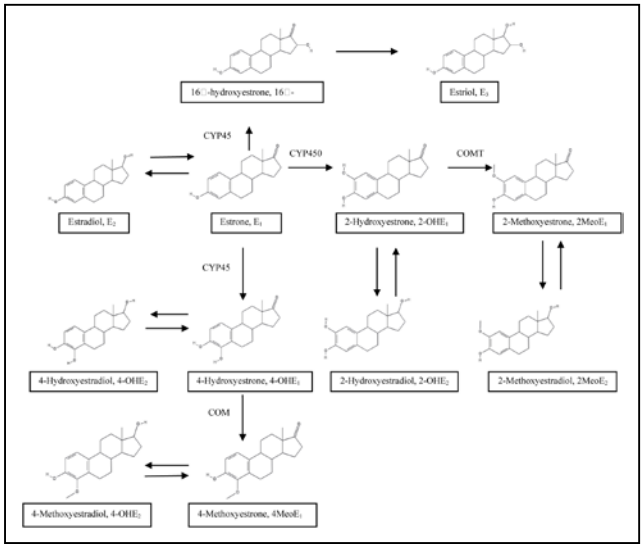
Mammographic density is a measure of the amount of fibroglandular tissue that appears on a mammogram. This measure is compared to the fat content in the breast tissue and is usually expressed as percent mammographic density (PMD). Mammographic density is a strong established risk factor for breast cancer.[10] It has been shown that high breast density (more than 75%) is linked with 4-6 times greater risk of breast cancer compared with no densities;[10-14] however, the involved mechanisms have not yet been fully elucidated. One of the proposed mechanisms through which mammographic density may modify breast cancer risk is by means of sex steroids.[15-17]

To date, the published data on the association between estrogens and mammographic density is scarce with mixed results. Current data is mostly limited to the circulating levels of estrogens and not the urinary hormones and their metabolites.[18-23] The purpose of this study is to test the hypothesis that estrogens and their metabolites are directly related to breast density. We also tested a secondary hypothesis that this effect is modified by the genetic variation in the COMT genotype within a cross-sectional analysis of postmenopausal women.

MATERIALS AND METHODS

Participants' eligibility and recruitment. The study sample used for this paper is a sub sample of a larger parent clinical trial "Green Tea and Reduction of Breast Cancer Risk." The parent trial is a randomized, double-blind, placebo-controlled trial. It aims to investigate the effects of green tea catechin intake on well-established biomarkers of breast cancer risk in 1000 healthy

Figure 1
Endogenous estrogen metabolism.



postmenopausal women at high risk of breast cancer due to dense breast tissue. Biomarkers that are studied include mammographic density, circulating and urinary reproductive hormones and their metabolites, insulin-like growth factor axis proteins, as well as oxidative stress. In addition, a possible differing effect of COMT genotype will be examined in all of the above biomarkers. Eligible participants for this sub sample analysis (n=208) were healthy postmenopausal women at increased risk of breast cancer due to high breast density, aged 50-70 years, non-smokers who do not regularly consume green tea (more than one cup of green tea per week) and have not taken hormone replacement therapy within six months of being screened for this study. Additional inclusion criteria include: alcohol consumption equal or less than 7 servings/week (one alcoholic serving size was defined as 12-oz of beer, 4-oz of wine, or 1.5-oz of liquor), BMI 19-35 kg/m^2, stable weight (less than 10 pounds change) for the past year, no previous diagnosis of breast cancer or proliferative breast disease, and no elevated levels of liver enzymes (above 1.5 times the upper limit of normal). Recruitment was conducted at the University of Minnesota Medical Center (UMMC), Fairview Southdale, and Fairview Maple Grove Breast Clinics in Minnesota.

The study radiologist supervised the reviews of the mammogram reports and the identification of those women with "heterogeneously dense" or "extremely dense" breasts. "Heterogeneously dense" breasts are approximately 51-75% glandular, and "extremely dense" breasts are >75% glandular. Once identified, an IRB approved recruitment letter was sent to prospective subjects explaining the intent of the study (IRB Approval Code Number: 0806M36121). If interested, the prospective subjects would call a screening phone number and subsequently attend an orientation session where the study was explained in more detail. Written informed consent was obtained at the end of each orientation session. Consented subjects then came for a screening in which blood was drawn for assessing the liver function status and COMT genotyping, and height and weight were measured to calculate BMI. Based on the results of the screening, final eligibility status of each participant was determined. If the participant was found eligible, she was scheduled for her baseline clinic visit, and she was randomized into either the green tea extract or placebo group. At baseline clinic visit, blood was drawn for measurement of all of the parent study biomarkers, and all subjects received their study supplements/ placebo. Data used for this paper is cross-sectional in nature, and only participant's baseline information, prior to beginning supplementation or placebo, was used in this analysis.

Urine collection and measurement and analysis of hormone levels. Subjects were instructed to collect urine for 24 hours prior to their morning baseline clinic visit times. They were asked to refrigerate the urine sample and avoid alcohol consumption during the 24 hour urine collection. Collected urine volumes were measured, and urine samples were aliquoted and stored at -80º within 2 hours upon receipt. Twelve urinary estrogens, including the primary estrogens and their metabolites, were analyzed by the liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay developed by Xu *et al*[24] as modified by our laboratory. All samples were measured in duplicate to ensure the accuracy of measurements.

COMT genotype. DNA was extracted from buffy coats of peripheral blood samples using a DNeasy Blood & Tissue Kit (QIAGEN Sample and Assay Technologies). TaqMan assays were developed for determining the COMT polymorphism gene variant using a TaqMan Drug Metabolism Genotyping Assay (Applied Biosystems).

Mammographic density measurement. The left cranio-caudal view of the digital screening mammograms of each participant was assessed to determine the PMD. Reading of the participants' mammograms was done by an experienced reader using the computer-assisted Madena method developed at the University of Southern California in which total breast area and dense area are quantified.[25] Percent density was calculated by dividing the dense area by the total area multiplied by 100. In order to measure the reliability of the percent density readings for quality control, approximately 10% of the total mammograms were read twice where the intra-class correlation coefficients of the duplicate readings were found to be more than 95%.

Statistical analyses. Urinary levels of estrogens and their metabolites were not normally distributed, so they were analyzed on the log-scale and are reported as medians and ranges. Association between the urinary estrogens and mammographic densities was determined using Spearman correlation coefficients adjusted for age, BMI, COMT

genotype and reproductive history factors. Data were analyzed using SAS software, version 9.2 (SAS Institute Inc.), and the value of P < 0.05 was considered statistically significant.

RESULTS

Selective characteristics of the study participants are summarized in Table 1. The mean +/- the standard deviation (SD) age of participant was 60.0 (5.1) years. Approximately 44% of the participants were either overweight or obese (BMI ≥ 25 kg/m^2). Most of the participants were white (96.1%) and non-Hispanic (98.1%), parous (73.3%), never smoked (67%); 83% had a previous history of using birth control, and 46% had a history of using HRT. The mean (SD) percent breast density was 32.3% (16.8). As expected in the Caucasian populations, about half of the participants possessed the COMT AG genotype, 18% had the GG and 27% had AA genotypes.

Table 2 displays the distribution of urinary estrogens and their metabolite concentrations and age and BMI. Parent estrogens comprised 28.2% of the total estrogens and metabolites, and were statistically significantly correlated with the hydroxylated estrogens including 2, 4, and 16-hydroxylated metabolites (P<0.0001 for all metabolite pathways). In contrast, estrogens and hydroxylated metabolites were negatively associated with age (P<0.05 for all of them) but non-significant positively related with HRT; however, their correlation direction with BMI was mixed and mostly non-significant (data not shown).

PMD was significantly and inversely associated with age and BMI (the Spearman correlation coefficient were -0.15 and -0.3, P= 0.03 and <0.0001, respectively). As shown in Table 2, the majority of primary estrogens and their metabolites, except 2-methoxy estradiol, were inversely associated with the PMD. However, this relationship was weak and never reached the statistical significance level. These results remained unchanged after further adjustment for age, BMI, reproductive and dietary factor covariates mentioned in Table 1. Further adjustment for the effect of COMT genotype on breast density and estrogen levels was performed, and no interaction was found for either the PMD or estrogens and their metabolites. Since age and BMI adjusted Spearman coefficients were not statistically significant for any estrogen metabolites, the associations between mammographic density and different hormones were no longer investigated based on their concentration categories.

Table 1
Baseline characteristics and reproductive histories of participants

Variable	n	Mean ± SD or %
Age, y	208	60.0 ± 5.1
BMI, kg/m^2	208	25.1 ± 4.0
≤24.9 (normal-underweight)	116	55.8%
25.0-29.9 (overweight)	70	33.6%
≥30.0 (obese)	22	10.6%
Race		
White	200	96.1%
Other	8	3.8%
Ethnicity		
Hispanic	4	1.9%
Non-Hispanic	204	98.1%
COMT genotype distribution		
GG	37	17.8%
AG	115	55.3%
AA	56	26.9%
Mammographic density, %	200	32.3 ± 16.8
Age at menarche, y	204	13.0 ± 1.6
Age at first live birth, y	148	32.2 ± 4.9
Number of live child birth	205	1.60 ± 1.2
Parity		
Nulliparous	55	26.7%
Parous	151	73.3%
Past use of birth control pills		
No	35	17.1%
Yes	170	82.9%
Family history of breast cancer		
No	122	58.7%
Yes	86	41.3%
HRT history		
No	110	53.7%
Yes	95	46.3%
Past smoker		
No	138	67.0%
Yes	68	33.0%
Current Alcohol intake		
No	47	22.7%
Yes	160	77.3%
Current black/green tea intake		
No	83	40.3%
Yes	123	59.7%
Current soy consumption		
No	136	68.0%
Yes	64	32.0%

NOTE: Values are presented as mean ± standard deviation (SD) for continuous variables, and n and % for categorical variables. BMI, body mass index; COMT, Catechol-O-methyl transferase; HRT, hormone replacement therapy.

Table 2
Urinary concentrations of estrogens and estrogen metabolites (µg/day), age, BMI, and corresponding Spearman correlations with PMD

Hormones	Median (range)[a]	Spearman correlation coefficient [b]	*P* value
Estrone	1.7 (0.2-21.3)	-0.13	0.07
Estradiol	0.4 (0.05- 7.9)	-0.11	0.14
Estriol	2.37 (0.004-22.9)	-0.05	0.51
2-Hydroxyestrone	1.79 (0.01- 25.5)	-0.03	0.64
4-Hydroxyestrone	0.23 (0.006- 4.7)	-0.07	0.29
16-Hydroxyestrone	0.18 (0.005- 4.1)	-0.01	0.89
2-Methoxyestrone	0.12 (0.003- 3.5)	-0.04	0.57
4-Methoxyestrone	0.02 (0.003- 0.5)	-0.04	0.55
2-Hydroxyestradiol	0.09 (0.002- 1.0)	-0.02	0.81
4-Hydroxyestradiol	0.01 (0.002- 1.1)	-0.13	0.07
2-Methoxy estradiol	0.04 (0.002- 0.8)	0.12	0.08
4-Methoxy estradiol	0.01 (0.003- 0.5)	-0.05	0.45
Total estrone and estradiol	2.15 (0.3- 29.2)	-0.12	0.09
Total estrogens and metabolites	6.98 (1.3- 85.9)	-0.08	0.28
Age (y)	59.8 (50.1- 71.2)	-0.15	0.03
BMI (kg/m^2)	24.2 (18.2- 38.6)	-0.3	<0.0001

NOTE: Values are presented as median (range) for baseline hormone levels, age and BMI. *P* values for baseline hormone levels are unadjusted for age and BMI.
[a] n=208
[b] n=200

DISCUSSION

The current literature is inconsistent regarding the associations between reproductive hormones in blood and/or urine and mammographic density. Most of the existing evidence comes from studies assessing the foregoing association in the blood, and their results vary from a null relationship to either a direct or inverse association.[18-22] In this study of postmenopausal women at increased breast cancer risk, higher urinary concentrations of primary estrogens and their metabolites were not associated with mammographic density. These null results remained unchanged with or without adjustment for age, BMI, hormone therapy history and COMT genotype.

To date, a total of eleven studies have evaluated the relationship between estrogens and mammographic density in postmenopausal women.[18-22, 26-30] Similar to the association direction found in this research, six previous studies[18-20, 28-30] have shown inverse associations between blood estrone or estradiol and PMD; however, three of these studies[19-20, 30] lost their statistically significant association after controlling for BMI. In contrast, three studies[21-22, 27] have found positive associations between circulating estrogens and PMD after adjustment of BMI. To the best of our knowledge, only one study[23] has investigated the association of urinary estrogens and their metabolites with PMD thus far. Fuhrman *et al* have reported positive correlations between estrone and estradiol with PMD; however, these associations did not reach statistical significance level. The only significant result found in their study was an inverse association between 2-methoxyestrone and 4-methoxyestrone and PMD. These conflicting findings in the studies can be due to several potential factors including different sample size, diverse study populations, difference in sex hormones analysis methods (e.g., immunoassays vs. LC-MS/MS), as well as restrictions in the present breast density quantifying procedures and techniques.

In the interpretation of our study results, some limitations should be considered. This study has a relatively small sample size compared with earlier studies, which had sample sizes up to 1400 participants. Another limitation is that this study was a cross-sectional study; therefore, inferring causal relationship is limited. In addition, urinary sex hormone concentrations were only measured at one time point which may not reflect the long-term levels and may not be an appropriate surrogate for breast tissue levels. Finally, a two month gap existed between the time of the mammogram and the baseline hormone measurements. We are unsure if this time gap may have influenced the hormonal measurements as compared to the breast density observed 2 months earlier. This study also has several strengths that should be noted. It is part of a large prevention clinical trial so we can update the current results with a much larger sample size of approximately 1000 postmenopausal women in the near future. We have also used digital screening mammograms, which have less limitation than using traditional film mammograms. Furthermore, the reader of the mammograms was blinded to the characteristics of the subjects. Mammograms were read twice, and both readings produced similar results. Lastly, analyzing the urinary estrogens and their metabolites was done using advanced LC-MS/MS methodology, which is very accurate and reproducible.

In conclusion, we found no evidence that urinary estrogens and their metabolites are associated with the mammographic density in healthy postmenopausal women at increased risk of breast cancer. Also, our findings do not suggest any significant interaction between COMT genotype and estrogens and mammographic density. These results should be evaluated and confirmed in larger studies.

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References

1. Siegel R, Naishadham D, Jemal A. Cancer Statistics 2013. *CA Cancer J Clin*. 2013; Jan 17. [Epub ahead of print].
2. Althuis MD, Fergenbaum JH, Garcia-Closas M, Brinton LA, Madigan MP, Sherman ME. Etiology of hormone receptor-defined breast cancer: a systematic review of the literature. *Cancer Epidemiol Biomarkers Prev*. 2004; 13: 1558-68.
3. Purohit A, Reed MJ. Regulation of estrogen synthesis in postmenopausal women. *Steroids*. 2002; 67:979-83.
4. Dorgan JF, Longcope C, Stephenson HE, Jr, et al. Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk. *Cancer Epidemiol Biomarkers Prev*. 1996; 5:533-9.

5. Toniolo PG, Levitz M, Zeleniuch-Jacquotte A, et al. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. *J Natl Cancer Inst*. 1995; 87:190-7.
6. Zeleniuch-Jacquotte A, Bruning PF, Bonfrer JM, et al. Relation of serum levels of testosterone and dehydroepiandrosterone sulfate to risk of breast cancer in postmenopausal women. *Am J Epidemiol*. 1997; 145:1030-8.
7. Lachman HM, Papolos DF, Saito T, Yu YM, Szumlanski CL, and Weinshilboum RM. Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. *Pharmacogenetics*. 1996; 6: 243-250.
8. Dawling S, Roodi N, Mernaugh RL, Wang X, and Parl FF. Catechol-O-methyltransferase (COMT)-mediated metabolism of catechol estrogens:comparison of wild-type and variant COMT isoforms. *Cancer Res*. 2001; 61: 6716-6722.
9. Yim DS, Parkb SK, Yoo KY, et al. Relationship between the Val158Met polymorphism of catechol O-methyl transferase and breast cancer. *Pharmacogenetics*. 2001; 11: 279-286.
10. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. *Cancer Epidemiol Biomarkers Prev*. 2006; 15: 1159-69.
11. Harvey JA, Bovbjerg VE. Quantitative assessment of mammographic breast density: relationship with breast cancer risk. *Radiology*. 2004; 230:29-41.
12. Boyd NF, Rommens JM, Vogt K, et al. Mammographic breast density as an intermediate phenotype for breast cancer. *Lancet Oncol*. 2005; 6:798-808.
13. Ursin G, Ma H, Wu AH, et al. Mammographic density and breast cancer in three ethnic groups. *Cancer Epidemiol Biomarkers Prev*. 2003; 12:332 – 8.
14. Byrne C, Schairer C, Wolfe J, et al. Mammographic features and breast cancer risk: effects with time, age, and menopause status. *J Natl Cancer Inst.* 1995; 87:1622 – 9.
15. So¨derqvist G. Effects of sex steroids on proliferation in normal mammary tissue. *Ann Med*. 1998; 30: 511–24.
16. Henderson BE, Ross RK, Bernstein L. Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Res*. 1988; 48: 246–53.
17. Key T, Appleby P, Barnes I, et al. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. *J Natl Cancer Inst*. 2002; 94: 606–16.
18. McCormack VA, Dowsett M, Folkerd E, et al. Sex steroids, growth factors and mammographic density: a cross-sectional study of UK postmenopausal Caucasian and Afro-Caribbean women. *Breast Cancer Res*. 2009;11:R38.
19. Verheus M, Peeters PH, van Noord PA, van der Schouw YT, Grobbee DE, van Gils CH. No relationship between circulating levels of sex steroids and mammographic breast density: the Prospect-EPIC cohort. *Breast Cancer Res*. 2007; 9:R53.
20. Tamimi RM, Hankinson SE, Colditz GA, Byrne C. Endogenous sex hormone levels and mammographic density among postmenopausal women. Cancer *Epidemiol Biomarkers Prev*. 2005; 14:2641–7.
21. Bremnes Y, Ursin G, Bjurstam N, Rinaldi S, Kaaks R, Gram IT. Endogenous sex hormones, prolactin and mammographic density in postmenopausal Norwegian women. I*nt J Cancer.* 2007; 121:2506–11.
22. Greendale GA, Palla SL, Ursin G, et al. The association of endogenous sex steroids and sex steroid binding proteins with mammographic density: results from the Postmenopausal Estrogen/ Progestin Interventions Mammographic Density Study. *Am J Epidemiol*. 2005; 162:826–34.
23. Fuhrman BJ, Brinton LA, Pfeiffer RM, et al. Estrogen metabolism and mammographic density in postmenopausal women: a cross-sectional study. *Cancer Epidemiol Biomarkers Prev*. 2012; 21:1582-91.
24. Xu X, Keefer LK, Ziegler RG, Veenstra TD. A liquid chromatography mass spectrometry method for the quantitative analysis of urinary endogenous estrogen metabolites. *Nat Protoc*. 2007; 2:1350–5.
25. Ursin G, Astrahan MA, Salane M, et al. The detection of changes in mammographic densities. *Cancer Epidemiol Biomarkers Prev*.1998; 7:43–7.
26. Warren R, Skinner J, Sala E, et al. Associations among mammographic density, circulating sex hormones, and polymorphisms in sex hormone metabolism genes in postmenopausal women. *Cancer Epidemiol Biomarkers Prev*. 2006; 15:1502–8.
27. Johansson H, Gandini S, Bonanni B, et al. Relationships between circulating hormone levels, mammographic percent density and breast cancer risk factors in postmenopausal women. *Breast Cancer Res Treat.* 2008; 108:57–67.
28. Boyd NF, Stone J, Martin LJ, et al. The association of breast mitogens with mammographic densities. *Br J Cancer*. 2002; 87:876–82.
29. Aiello EJ, Tworoger SS, Yasui Y, et al. Associations among circulating sex hormones, insulin-like growth factor, lipids, and mammographic density in postmenopausal women. *Cancer Epidemiol Biomarkers Prev*. 2005; 14:1411–7.
30. Sprague BL, Trentham-Dietz A, Gangnon RE, et al. Circulating sex hormones and mammographic breast density among postmenopausal women. *Horm Cancer*. 2011; 2:62–72.